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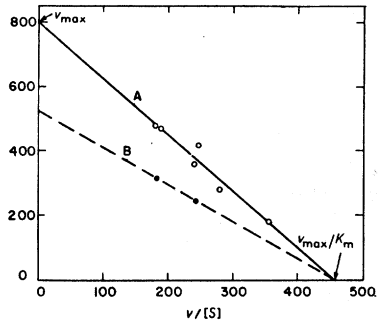
The glucose oxidase of honey. IV. Some addition observations

During the course of our previous studies[1-4] on honey glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4) we had encountered certain points which required further investigation and had left some questions unanswered. Several of these topics have now been studied and the findings are reported here. Evidence is given for a coupling or uncompetitive inhibition by fructose. Hydrogen peroxide had little effect on initial velocity, while ascorbic acid caused an acceleration. L-Glucose was without activity as a substrate.

The glucose oxidase was prepared as previously described[2,3]. It had a specific activity of 279 units/mg (see ref. 2 for definition of units). The D-glucose substrate was a solution of equilibrium glucose (α and β) made from α-D-glucose (J. T. Baker Chemical Company*). L-Glucose was obtained from Nutritional Biochemicals Corporation, D-fructose from Dawe's Laboratories, Inc., L-ascorbic acid from Eastman Organic Chemicals, and hydrogen peroxide from J. T. Baker Chemical Company. All experiments were run in O_2 at 37° using standard Warburg manometric procedures.

Fructose effect. In earlier work it had been noted that the presence of fructose in the glucose oxidase system sometimes led to increased H_2O_2 production[1], while at



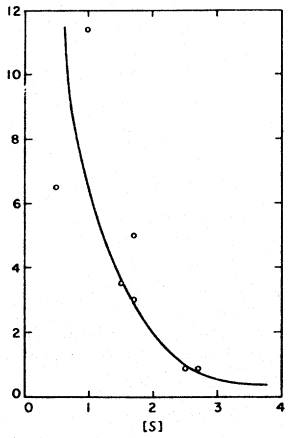


Fig. 1. Plot of velocity–substrate data. In A, the complete system contained: 0.5 to 2.7 ml 3.5 M glucose in 0.2 M sodium phosphate (pH 6.1), and enough of the same buffer to total 3.4 ml in the main-space; 0.1 ml (419 units) enzyme preparation in a side-arm sac; 0.2 ml 10% KOH in the center well. In B, the system was the same except 1.7 M fructose was present in the main-space at the 1.7 M and 1.0 M glucose levels. Blanks were run without enzyme, without substrate. v, initial velocity in μmoles/min; [S], substrate concentration in M; v_{max}, maximum velocity; K_m, Michaelis constant.

Fig. 2. Plot of transposed velocity–substrate data. Conditions same as in Fig. 1, except that fructose concentration was varied from 0.1 to 2.2 M; the combined sugar concentrations never exceeded 3.4 M.

* Mention of company or trade names does not imply endorsement by the Department over others of a similar nature not named.

other times it caused an inhibition[2]. This seemingly contradictory effect was investigated using varying concentrations of glucose (0.5 to 2.7 M) and fructose (0.1 to 2.2 M). If we take the velocity–substrate data for glucose alone and plot v against $v/[S]$ (according to AUGUSTINSSON[5]) we obtain the usual straight line (Fig. 1, Line A) from which v_{max} and K_m can be calculated. However, if we now plot the results from two of these experiments in which 1.7 M fructose was also present, Line B, which intersects A at v_{max}/K_m, is obtained. This would suggest an inhibition by fructose of the type classified by WEBB[6] as "coupling or uncompetitive inhibition". Further evidence for this is found by taking all of the fructose–glucose data and, since the concentrations of both had been varied, transposing and plotting them by the method of HUNTER AND DOWNS[7] as seen in Fig. 2. A hyperbolic curve of this type is found only in the rare case of coupling or uncompetitive inhibition[6], wherein the inhibitor combines only with the enzyme–substrate complex, never with the enzyme alone. According to WEBB[6] this gives rise to circumstances in which the inhibitor can actually cause activation as well as inhibition. It would appear that the unusual behavior of fructose observed in the glucose oxidase system was due to this type of phenomenon.

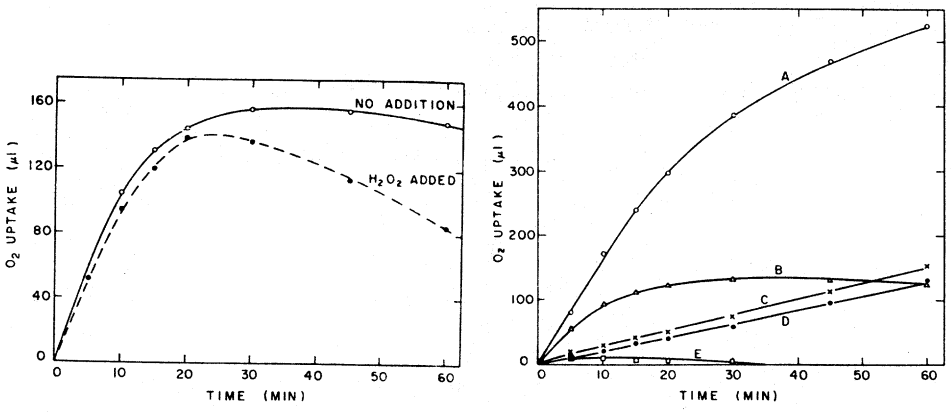


Fig. 3. Effect of added H_2O_2 on glucose oxidase system. Complete system contained: 2.7 ml 3.5 M glucose in 0.2 M sodium phosphate (pH 6.1), 0.6 ml of the same buffer, 0.1 ml H_2O_2 solution (82 mg/ml) or 0.1 ml H_2O in the main-space; 0.1 ml (419 units) enzyme preparation in a side-arm sac; 0.2 ml 10% KOH in the center well. Blanks were run without enzyme, without substrate. Final concentration of H_2O_2 was 2340 µg/ml.

Fig. 4. Effect of ascorbic acid on the glucose oxidase reaction. Complete system contained: 1.5 ml 3.5 M glucose in 0.2 M sodium phosphate (pH 6.1), and 1.9 ml of the same buffer in the main-space; 0.1 ml (419 units) enzyme preparation in a side-arm sac; 0.2 ml 10% KOH in the center well. In flasks where ascorbate was added, this was done by tipping in 6 mg of solid L-ascorbic acid from another side-arm sac. Blanks were run without enzyme, without substrate. A, enzyme + glucose + ascorbate; B, enzyme + glucose; C, glucose + ascorbate; D, ascorbate only; E, enzyme + ascorbate. A and E were corrected for autoxidation.

H_2O_2 effect. In following the oxidation of glucose in this enzyme system, a decrease in velocity had always been observed after the initial portion of the progress curve[4]. While this was assumed to be due to H_2O_2 accumulation, it had not been verified. (That this decrease was not caused by inhibition by the other reaction product, gluconolactone, was already established[2].)

A series of experiments was conducted in which H_2O_2 was added initially to the glucose oxidase system. At a concentration (234 μg/ml) comparable to that expected after 60 min of glucose oxidation there was no significant effect. At 10 times this concentration (2340 μg/ml) the result was, at most, only a 10% inhibition of the initial velocity, but a considerable decline in rate after the initial portion (Fig. 3). This was seen even when Na_2S had been added to inhibit any catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) activity[8]. Examination of blanks showed that much of the H_2O_2 had reacted with the glucose. The extent of this side reaction was dependent upon the glucose concentration, but could not be accounted for by the amount of O_2 produced. More than a simple reduction of H_2O_2 was probably involved[9]. Due to this complication, it was impossible to get a clear picture of the H_2O_2 effect. If inhibition does occur, it is not appreciable during the initial portion of the reaction and thus has little effect on initial velocity.

L-*Glucose.* Since the honey glucose oxidase had been shown[4] to require large concentrations of D-glucose (2.7 M) for optimal activity and yet displayed rather poor affinity for it (K_m, 1.55), there had always been a question of whether this actually was the best substrate, even though many others had been tried[2]. With the discovery of a report by BARBER AND HASSID[10] in which certain plant seedling extracts were able to oxidize L-glucose to L-gluconic acid, it was decided to test L-glucose as a possible substrate in the honey system. The enzyme was unable to utilize the L-form, indicating a clear specificity for the D-form of glucose.

Ascorbic acid effect. In view of the uncertainty of the presence of ascorbic acid in honey[11–13] and its possible reaction, if present, with an oxidation system, the effect of ascorbic acid on the glucose oxidase reaction was examined. The dramatic result is shown in Fig. 4. Addition of ascorbic acid (Curve A) caused a tremendous increase in O_2 uptake (corrected for autoxidation) over the usual glucose oxidation rate (Curve B). The autoxidations (Curves C and D) progressed at a much slower, but linear rate. Curve E indicates that there was no significant ascorbate oxidase (L-ascorbate:O_2 oxidoreductase, EC 1.10.3.3) activity in the enzyme preparation and thus the oxidation of the ascorbic acid was probably due to the following reactions:

$$\text{Glucose} + O_2 \rightarrow \text{gluconolactone} + H_2O_2 \qquad (1)$$

$$H_2O_2 + \text{ascorbate} \xrightarrow{Cu^{2+}} \text{dehydroascorbate} + 2H_2O \text{ (ref. 14)} \qquad (2)$$

This removal of H_2O_2, of course, provides considerable acceleration to the first step. The autoxidation reaction causes O_2 uptake by the following mechanism:

$$\text{Ascorbate} + O_2 \xrightarrow{Cu^{2+}} \text{dehydroascorbate} + H_2O_2 \text{ (ref. 14)} \qquad (3)$$

The H_2O_2 is then removed as in reaction (2).

The effect of ascorbic acid on the glucose oxidase system is, therefore, as a powerful activator, but this is by way of product removal and not, presumably, due to any action on the enzyme itself. Also, it would seem highly unlikely that ascorbic acid (in the reduced form) would be found in a honey containing significant glucose oxidase activity. In converse, a honey high in ascorbic acid would not be expected to show a significant peroxide accumulation due to glucose oxidase activity.

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ABNER I. SCHEPARTZ

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